Carbon monoxide- and oxygen-reacting haemoproteins in the mitochondrial fraction from the soil amoeba *Acanthamoeba castellanii*

Studies at subzero temperatures

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1. Mitochondria-enriched fractions of the soil amoeba *Acanthamoeba castellanii* contained four haemoproteins that in their reduced forms reacted with CO to give photodissociable CO complexes; these were cytochromes a_3 , a_{614} , b - and c -type cytochromes. 2. Non-photodissociable oxygen-containing compounds were formed at temperatures between -130 and -150°C after photodissociation of CO in the presence of $200\ \mu\text{M-O}_2$. 3. Electron transport, indicated by the oxidation of cytochromes $a + a_3$ and cytochrome c , did not occur until the temperature was raised to -80°C .

Mitochondrial respiration mediated by cytochrome $a + a_3$, the terminal oxidase of the phosphorylating electron-transport chain, is competitively inhibited by CO (Chance, 1957). The occurrence of other CO-binding terminal oxidases is widespread in bacteria (Castor & Chance, 1959), but is rather unusual in mitochondria (Lloyd & Edwards, 1978).

The reaction of haemoproteins with CO may assist in their identification, but may also be indicative of those components that also react with O_2 . The reaction of cytochrome oxidase with O_2 results in the formation of spectrophotometrically distinct complexes, and low-temperature studies have shown that some of these are capable of oxidizing cytochrome c (Chance *et al.*, 1978*a*). The technique of trapping O_2 intermediates at low temperatures (Chance *et al.*, 1975*a*) has successfully been employed to identify intermediates in the reaction of O_2 and cytochrome oxidase, whether solubilized (Chance *et al.*, 1978*b*), in intact mitochondria (Chance *et al.*, 1975*b,c*) or in intact cells of *Schizosaccharomyces pombe* (Poole *et al.*, 1979*b*); intermediates in the reaction of cytochrome o with O_2 in intact *Escherichia coli* have also been identified by this method (Poole *et al.*, 1979*a*).

Mitochondria-enriched fractions prepared from the soil amoeba *Acanthamoeba castellanii* contain three CO-binding haemoproteins other than cyto-

chrome a_3 (Edwards & Lloyd, 1980). The present study describes further characterization of cytochrome a_{614} , b - and c -type cytochromes, all of which react with CO and O_2 at subzero temperatures.

Experimental

Maintenance and growth of the organisms

Acanthamoeba castellanii was maintained, grown axenically with shaking at 30°C , and counted, as described previously (Edwards *et al.*, 1977).

Harvesting and breakage of the organism, preparation of mitochondrial fractions and submitochondrial particles

Methods for harvesting and homogenization of organisms and preparation of mitochondria-enriched fractions were as described by Edwards & Lloyd (1978). Submitochondrial particles were prepared by treatment with ultrasound (Edwards & Lloyd, 1980).

Low-temperature flash photolysis

Suspensions of mitochondria or submitochondrial particles (10–20 mg of protein/ml) were incubated with 15% (v/v) ethylene glycol and sodium succinate (10 mM) or NADH (1 mM) in plastic cuvettes of 4 mm light-path for 5 min at 20°C . These anaerobic suspensions were bubbled with CO for 2 min (final concn. 1.2 mM), followed by a further 5 min incubation in the dark at room temperature. The cuvette was then cooled to -25°C

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in a solid-CO₂/ethanol mixture and allowed to equilibrate for 5 min. At this point in the procedure some suspensions were oxygenated by stirring vigorously with a stainless-steel coil that fitted the cuvette. Stirring at 2 Hz for 20 s introduced 200 μ M-O₂ (Chance *et al.*, 1975a). Cuvettes were then rapidly transferred to a freezing mixture at -80°C where they were stored for up to 30 min. Transfer to the cuvette compartment of the scanning dual-wavelength spectrophotometer (Chance *et al.*, 1972) was followed by equilibration to the temperature at which photolysis and observation of reaction kinetics was performed.

Illumination of the sample by the measuring light was prevented during thermal equilibration. Except where stated otherwise, baseline corrections (spectra of CO-ligated anaerobic cell suspensions) were stored in a digital memory. The cuvette was cooled by a stream of cold N₂ gas and sample temperature was monitored and maintained by an adjacent thermocouple (Chance *et al.*, 1975a) before flash photolysis (up to six flashes of a 20 J xenon flash-lamp).

Results

Photodissociation at temperatures lower than -140°C

A suspension of mitochondria was allowed to respire to anaerobiosis in the presence of 10 mM-succinate at 20°C and was then bubbled with CO for 2 min. After cooling to -145°C the spectrum of this preparation was scanned and stored in the instrument memory of a dual-wavelength scanning spectrophotometer with the reference wavelength set at 500 nm. A baseline (recorded before flash photolysis) represents a CO-reduced-minus-CO-reduced spectrum (Fig. 1). Flash photolysis (four flashes) was monitored by spectrophotometric scans at 90 s intervals (reduced-minus-Co-reduced spectra), which indicated the presence of several CO-reacting species. Minima at 433, 543 and 593 nm represent loss of absorption due to photolysis of the cytochrome *a*₃-CO complex; a maximum at 450 nm and a shoulder at about 606 nm corresponded to the absorption of unliganded cytochrome *a*₃. Other features included maxima at 553, 565 and 616 nm,

Fig. 1. Repetitive wavelength scanning of the reaction of haemoproteins in a mitochondrial fraction of *A. castellanii* with CO at -145°C

The spectrum of a suspension of CO-ligated succinate-reduced mitochondria to which O₂ had not been added was scanned and stored in the digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 500 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The first scan, before photolysis, yielded the baseline (---), which represents a CO-reduced-minus-CO-reduced spectrum. Photolysis at -145°C preceded five scans (numbered 1 → 5) at intervals of 90 s. Scanning was from right to left at about 3.5 nm/s. Absorbance increments and the wavelengths (in nm) of distinctive spectral features are shown.

suggesting the presence of *c*-type, *b*-type and another *a*-type photodissociable CO-binding cytochrome respectively. Five successive scans gave no detectable change in the spectrum, showing that recombination of CO after photolysis was insignificant at this low temperature for all binding pigments present.

Changes after photodissociation of CO in the presence of $200\mu\text{M-O}_2$ at -150°C are shown in Fig. 2. In this experiment, photodissociation of bound CO with four flashes preceded acquisition of the stored spectrum and recording of the baseline (reduced-minus-reduced spectrum). Six successive spectrophotometric scans recorded the progress of ligand binding. As CO binding is undetectable at this temperature, the spectral changes are indicative of liganding of O_2 . This was confirmed by the non-photodissociability of the complexes formed (results not shown). Disappearance of reduced cytochrome a_3 (minimum at 449nm) was accompanied by the formation of an oxygen-containing compound (maximum at 429nm, shoulder at 443nm; Chance *et al.*, 1975*b,c*); corresponding changes in the

α -absorption region gave a maximum at 591nm and a minimum at 599nm. Another oxygen-reacting component was evident at wavelengths greater than 600nm (maximum 607nm, broad minimum at around 614nm). Reaction of *c*- and *b*-type cytochromes (maxima at 554 and 565nm, minima at 547 and 560nm) was also observed to occur under these conditions.

Progress of the formation of oxygen-containing compounds was also monitored in submitochondrial particles at -133°C (Fig. 3). Whereas treatment with ultrasound releases the CO-reacting *b*-type cytochrome from the mitochondria, Edwards & Lloyd (1980) have previously shown that submitochondrial particles retain a CO-reacting *c*-type cytochrome. In this case the reductant was 1mM-NADH and the baseline was plotted before photodissociation (CO-reduced-minus-CO-reduced spectrum). Complex spectral changes gave progressive shifts to produce a shoulder of about 443nm in addition to the trough at 429nm initially observed. A double trough (with minima at 585 and 596nm) was also eventually produced in the region

Fig. 2. Reaction of mitochondrial haemoproteins of *A. castellanii* with O_2 at -150°C

A suspension of CO-liganded succinate-reduced mitochondria into which O_2 had been stirred at -25°C was cooled to -150°C then photolysed. A spectrum was immediately scanned and stored (reference wavelength 500nm). Subsequent scans are difference spectra with the stored spectrum subtracted. The broken line (---) is the first spectrum recorded after photolysis. Six spectra (1→6) were subsequently recorded at intervals of 90s. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

Fig. 3. Reaction of haemoproteins in submitochondrial particles from *A. castellanii* with O_2 at $-133^\circ C$. A suspension of CO-liganded NADH-reduced mitochondria into which O_2 had been stirred at $-25^\circ C$ was cooled to $-133^\circ C$. A spectrum was scanned and stored. The first scan, before photolysis, yielded the baseline (----). Presentation of results is identical with that in Fig. 1.

Fig. 4. Recombination of CO with haemoproteins of a mitochondrial fraction of *A. castellanii* at $-96^\circ C$. Conditions and presentation of results are otherwise identical with those of Fig. 1.

of α -absorption due to α -type cytochromes. Reaction with c -type cytochrome gave a maximum and minimum at 549 and 543 nm respectively.

Compounds formed in the presence of O_2 were not significantly photolysed by four flashes of the xenon lamp. A control experiment, on the other hand, indicated that the spectrally distinct inter-

mediates formed in the absence of O_2 were immediately photolysed under similar conditions.

Photolysis over the temperature range -90 to $-120^\circ C$

Further evidence for the presence of four CO-binding cytochromes in *A. castellanii* was pro-

Fig. 5. Electron transport in mitochondrial fraction of *A. castellanii* at -63°C

The methods used and presentation of results are identical with those of Fig. 2, except that the reaction was initiated and observed at -63°C in the presence of $200\ \mu\text{M-O}_2$.

vided by a study of the kinetics of photodissociation of the CO-liganded species at -118°C after a single flash. Dissociation of all four CO complexes was again observed (cytochrome a_3 -CO at 430 and 587 nm, cytochrome a_{614} -CO at 608 nm, c - and b -type cytochrome ligands at 553 and 567 nm (results not shown). When this preparation was photolysed at -96°C in the absence of O_2 (four flashes), rapid recombination of all four species with CO was observed (Fig. 4).

Electron transport after photolysis at temperatures higher than -80°C

When flash photolysis was performed at temperatures higher than -80°C in the presence of $200\ \mu\text{M-O}_2$, changes in absorption spectra were observed that may be ascribed to electron transport. Oxidation of cytochrome $a + a_3$ (at 590–605 nm) cytochrome b (at 565 nm), and cytochrome c (at 549 nm) was evident in successive scans in the α -absorption region at -63°C (Fig. 5).

Discussion

These results confirm and extend the previous observations of the presence of a diversity of CO-reacting cytochromes in washed mitochondrial fractions prepared from *A. castellanii* (Edwards & Lloyd, 1980); reaction of four spectrophotometrically distinguishable species with O_2 has now also been observed. Although it is probable that the reaction of O_2 with these cytochromes involves the

formation of intermediary complexes, identification of these and assignment of spectral features is difficult in view of the complexity of the system. Also unusual is that, whereas in mammalian mitochondria cytochrome c oxidation is very slow and indistinct at -78°C (Chance *et al.*, 1977), electron transport is evident in mitochondria of *A. castellanii* at -80°C .

The reaction of cytochrome $a + a_3$ with O_2 under the experimental conditions employed here results in the formation of spectrophotometrically distinct complexes that are functional in electron transport. These are compound A [which may be detected in the temperature range -105.8 to -89°C (Chance *et al.*, 1977)] characterized by an absorption maximum at 591 nm and a minimum at 611 nm, and compound B, which has an absorption minimum at 609 nm (Chance *et al.*, 1975a,b). At temperatures higher than -90°C , compound B is formed from compound A (Chance *et al.*, 1977).

Cytochrome o , a functional terminal oxidase in some bacteria (Castor & Chance, 1959), also reacts with O_2 in intact *Escherichia coli* under the conditions employed here to give intermediary complexes (Poole *et al.*, 1979a). In the temperature range of -79 to -102°C , intermediate(s) are formed that are spectrally similar to the reduced cytochrome o -CO complex, but distinguished by the following observations: (i) the CO-liganded oxidase is readily photodissociable, whereas the O_2 intermediate is not; (ii) the velocity of recombination of reduced oxidase and O_2 is greater than the rate of recombination with CO (Poole *et al.*, 1979a).

Identification of similar intermediates in mitochondrial fractions of *A. castellanii* and resolution of possible sequences of reactions with O_2 may only be possible in preparation of purified individual cytochromes. Although at least four different components have been demonstrated to react with CO and O_2 , there is no evidence as yet that three of these are functional in electron transport under physiological conditions. At low temperature, photolysis and ligand exchange with O_2 occurs with oxidases that are superficially very diverse; this observation supports the suggestion that there may be only one kind of first-, second- and third-shell structure that is capable of reacting with O_2 and reducing it in subsequent steps (Chance & Waring, 1978). Identification of functional terminal oxidases in this organism can only be ascertained by determination of the photochemical action spectra for the release of inhibition of respiration on photolysis of CO-liganded haemoproteins.

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